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Failure to Inhibit In Vitro or In Vivo Acetycholinesterase with Botulinum Toxin Type A

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An attempt has been made to replicate an earlier finding that type A botulinum toxin can inhibit the in vitro activity of acetylcholinesterase. Two methods of enzyme assay were employed, but with neither technique were we able to reproduce the finding of in vitro enzyme inhibition. In fact, an examination of the data from the previous report leads us to question the possibility of the observations that were given. Furthermore, an investigation was carried out to determine if botulinum toxin can exert an inhibiting effect on acetylcholinesterase that is situated in the biological tissue. The answer again is negative. The experimental observations, coupled with several mathematical computations, do not support the notion that botulinum toxin is an acetylcholinesterase inhibitor.

The cholinolytic effect of botulinum toxin is believed to be caused by the action of this poison in suppressing the presynatic release of acetylcholine (ACh; 1). Study supporting this theory has originated almost entirely from observations on the peripheral nervous system or on in vitro preparations. Two recent reports have noted that in the central nervous system (CNS) of mammals suffering botulinal intoxication there results a dramatic increase in the brain stores of ACh (3, 7). It was postulated that the increased levels of ACh were indicative of the poison's acting on the CNS in a manner similar to that on the peripheral nervous system; thus, the suppressed release of ACh caused a damming and internal buildup of neurohumor.

A recent study indicates that botulinum toxin, in a physical system that contains no biological tissue, is able to inhibit acetylcholinesterase (AChE; 6). The observation raises the question of whether the previously noted CNS increases in tissues levels of ACh reflect (i) suppressed release of ACh which is thereby protected from hydrolysis, or (ii) normal metabolic production of ACh during inhibition of its degrading enzyme. To choose between these two alternatives, we proceeded to replicate the above finding of an AChE inhibition by botulinum toxin and additionally to determine if the effect was demonstrable in vivo or in vitro.

MATERIALS AND METHODS

Botulinum toxin type A was kindly supplied by Edward J. Schantz, U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md. Crystalline toxin was stored in a phosphate-buffered gelatin solution, adjusted to *p*H 6.8, and then diluted with an equal volume of glycerol. Samples for testing were serially diluted in normal, unbuffered saline. AChE (electric eel) was obtained from Schwarz Bio Research, Inc., Orangeburg, N.Y. The enzyme had a specific activity of 1,000 units/mg, a unit being defined as that amount of enzyme which hydrolyzes 1 μmole of ACh per min at 27 C. Indophenyl acetate (IPA) was purchased from Mann Research Laboratories, Inc., New York, N.Y. Acetylthiocholine iodide was obtained from Calbiochem, Los Angeles, Calif., and 5,5′-dithiobis (2-nitrobenzoic acid) was procured from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Two methods of assay for AChE were employed, that of Kramer and Gamson (4) and that of Ellman, Courtney, Andres, and Featherstone (2). It is the former technique that was used by Marshall and Quinn (6) in reporting enzyme inhibition by toxin. The latter technique, which is two orders of magnitude more sensitive than the Kramer and Gamson method, is the assay routinely used in our laboratories.

The temperature and duration of assay were identical to those previously used (6). The toxin concentration was varied by steps of 10, from 10^{-2} to 10^{-8} mg/ml for both assays. Optical density changes due to enzyme activity were followed with a Gilford model 2000 recording spectrophotometer.

In vivo studies were performed on female Sprague

Dawley rats weighing 200 to 250 g. Cortices were assayed for both AChE and butyrylcholinesterase (BuChE) by a modification of the Ellman et al. technique (2). To measure AChE, acetylthiocholine (1.6×10^{-4} M) was used as a substrate, and 10- (2-dimethylaminopropyl) phenothiazine (2.5×10^{-5} M) was used as a BuChE inhibitor. To measure BuChE, butyrylthiocholine (1.0×10^{-4} M) was used as a substrate, and 1,5-bis (*N*-allyl-*N*,*N* dimethyl-4-ammoniumphenyl) pentan-3-one dibromide (5.0×10^{-7} M) was used as an AChE inhibitor. The BuChE inhibitor, known as Promethazine, can be obtained from Wyeth Laboratories, Philadelphia, Pa. The AChE inhibitor, labeled BW 284c51, was generously supplied by Burroughs Wellcome & Co., New York, N.Y.

RESULTS AND DISCUSSION

In the initial experiment, using the Kramer and Gamson assay, the concentration of reactants, as suggested in the earlier publication, were AChE = 0.17 unit/ml (Marshall and Quinn state a value of 5.0 units/ml. However, their number is based on a unit of enzyme activity being that amount which hydrolyzes 10.1 μg of ACh/min at 37 C, thus the difference in units per milliliter. It must be emphasized, nonetheless, that although there is a disparity in terms of "units" of enzyme per milliliter, the activity per milliliter in both studies is identical); botulinum toxin = 3.3×10^{-8} mg, and IPA = 1.3×10^{-6} M. Under these conditions, we were unable to detect any inhibition of AChE whatsoever; for in fact we were unable to detect a measurable reaction rate of IPA hydrolysis. The inactivity was manifest both in the presence and in the absence of botulinum toxin; thus, enzyme inhibition could not have been the determinant. Additionally, AChE was increased and decreased, in separate tests, by a factor of 10, neither alteration producing a definitive reaction rate.

This puzzling result prompted us to investigate the simple kinetics of the AChE-IPA reaction and further to delineate the conditions under which one might expect to witness a measureable hydrolysis rate. For these purposes, an absorption spectrum of indophenol, the hydrolysis and spectrophotometrically active product of IPA, was taken. The compound has a major peak at 625 nm, and at this peak, the molar absorptivity coefficient is 8,160 (Fig. 1). With these data, one may use the Beer-Lambert law to ascertain the maximal optical density change that any given concentration of IPA will produce when completely converted by enzyme to indophenol. With a molar absorptivity coefficient of 8,160, a path length of 1 cm, and a concentration of IPA of 1.3×10^{-6} M, the maximal optical density change possible is little more than 0.01, or approximately one-fiftieth of what Marshall and Quinn report seeing.

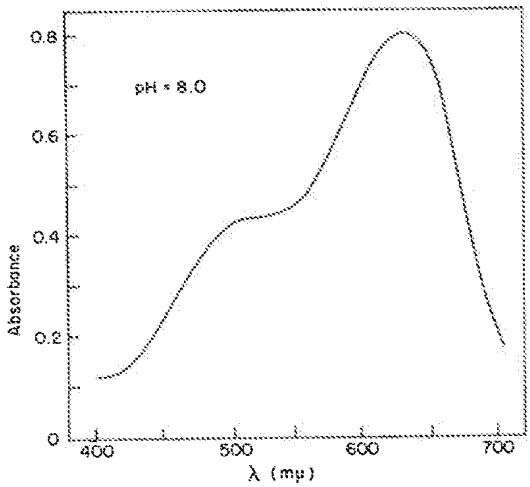

FIG. 1. *Absorbtion spectra of 1.3×10^{-4} M indophenol. The reading was made at 22 C in pH 8 phosphate buffer. There is a major peak at 625 nm of 8,160; it is this value which is used to determine by the Beer-Lambert Law the maximal optical density change possible following enzymatic hydrolysis of indophenyl acetate.*

Boosting the IPA concentration by a factor of 10 would have permitted visualization of the reaction, but even this would be insufficient to permit kinetic studies. Consequently, we raised the IPA concentration 100-fold, and then again attempted replication of the AChE inhibition by toxin. No dramatic indication of inhibition was apparant, regardless of the conditions under which the experiment was performed (Table 1). A minimal inhibition of less than 5%, insignificant due to variability, was all that was obtained, but certainly nothing approaching the previous figure of approximately 25% inhibition (6).

To ensure that we were not in error by using the older assay technique, the Ellman et al. method was used. Even when the toxin concentration was raised to 10^{-3} mg/ml, we could see no inhibition of enzyme activity.

At this point, it may be useful to introduce a series of mathematical computations. AChE, according to most recent estimates, has a molecular weight of about 250,000 and specific activity of 750 mmoles ACh hydrolyzed per hr per mg of enzyme (5). By use of these data, plus Avagadro's Law, one may calculate that there are approximately 1.8×10^8 molecules of ACh hydrolyzed per hr per molecule of AChE. Marshall and Quinn report use of 5 units of enzyme/ml or 15 units/assay. Taking into consideration the value

TABLE 1. *Resume of conditions under which the Kramer and Gamson assay technique was employed*[a]

Determination	AChE			Toxin	IPA	
	0.017 (unit/ml[b])	0.17	1.70	10^{-2} to 10^{-9} mg[c]	1.3×10^{-4} M	1.3×10^{-5} M
AChE (units)				0.017 to 1.70 at 3.3×10^{-6} mg of toxin 0.17 otherwise	0.17	0.017 to 1.70
Toxin (mg)	3.3×10^{-8}	10^{-8} to 10^{-3}	3.3×10^{-6}		10^{-3} to 10^{-8}	3.3×10^{-6}
IPA (M)	10^{-6}	10^{-4} to 10^{-8} (values $\times$ 1.3)	10^{-6}	1.3×10^{-6} at 3.3×10^{-6} mg of toxin 1.3×10^{-4} otherwise		

[a] The table indicates the concentrations of enzyme, toxin, and substrate used in various experimental conditions. The information should be read vertically; thus, with 0.017 unit/ml of AChE we used 3.3×10^{-6} mg of toxin and 10^{-6} M IPA. All conditions were run at both 25 and 37 C.

[b] Unit refers to the amount of enzyme which hydrolyzes 1 μmole of ACh/min at 27 C.

[c] Number refers to milligram of toxin per assay.

of their unit, one may calculate that these investigators used sufficient enzyme to hydrolyze 3.8×10^{10} molecules of ACh/hr in the presence of 3.3×10^{-6} mg of toxin and one-tenth that amount in the presence of 3.3×10^{-10} mg of toxin. This represents 2×10^{11} and 2×10^{10}, molecules, respectively, of AChE per assay.

Botulinum toxin has an approximate weight of 900,000. Consequently, 10^{-6} mg of toxin per 3 ml reaction cuvette means approximately 2×10^{9} molecules of toxin; similarly, 10^{-10} mg means approximately 2×10^{5} molecules of toxin.

It is interesting to pair the data derived from these computations. According to the earlier report (6), 2×10^{5} molecules of toxin can produce nearly 25% inhibition of 2×10^{10} molecules of AChE, and 2×10^{9} molecules of toxin can produce the same order of inhibition of 2×10^{11} molecules of AChE. An enzyme inhibitor-enzyme ratio of 1:100,000, with a resulting 25% inhibition, is most phenomenal. We were unable to obtain the result. Furthermore, by use of the Ellman et al. technique, with 10^{-6} mg of toxin, the toxin to enzyme ratio was nearly 1:1. No positive result, i.e., enzyme inhibition, was obtained here.

Two factors may have contributed to the failure to replicate the earlier reported result of inhibition. First, our investigation was performed by using glass apparatus, whereas the earlier study used plastic ware. This may have produced a difference in that the glassware could conceivably bind, by polar charges, the small quantities of toxin. Indeed, a difference in result depending on whether glass or plastic ware is used has been noted (R. Marshall, *personal communication*). However, the importance of this observation is questionable, because all cuvettes used for spectrophotometric study are made of glass. Second, all toxin maintained in our laboratory is stored in phosphate-buffered gelatin solution with glycerol, but the earlier investigators used unbuffered saline. It is conceivable that bonds between the toxin and either the gelatin or the phosphate shielded the groups that might otherwise react with AChE.

In this context, there are three notes which should be made on the data presented in the Marshall and Quinn report. First, the optical density changes for all the control preparations are identical. This could mean that the investigators enjoyed remarkable success in running the various control preparations. More likely, a single control or control condition was run, and this value was used in calculating all the data. Such a method is faulty, for one should run a control or standard for each condition. Thus, we would expect to see differences between those preparations which contained, and those which did not contain, antitoxin; we would even expect to see small differences among the various antitoxin preparations. Second, the data show an identical optical density change for a preparation containing 5 units of enzyme/ml and a preparation containing 0.5 unit/ml, the latter reaction running 2.5 times longer than the former. The observation is unlikely. Finally, the values given must obviously be the means of the several experiments, but no standard deviations are indicated. Conse-

quently, the reader cannot compare the effectiveness of inhibition at different toxin concentrations.

In any event, we proceeded to examine the possibility that botulinum toxin could inhibit AChE when the enzyme was still situated in biological tissue. The examination had two approaches. In the first, toxin (10^{-4} mg) was administered into the lateral tail vein of rats. When the animals were moribund (the moribund state of the animals showed dramatically that the molecular moiety responsible for the poison's toxicity was not shielded), they were decapitated and decorticated. The cortices were analyzed for AChE and BuChE by the modified Ellman et al. technique (2). In the second approach, cortices were removed from rats, and 10^{-3} mg of toxin was added to the cortex homogenate. This second approach was employed to circumvent the criticism that botulinum toxin does not always successfully invade the CNS. Figures 2 and 3 show the results of these in vivo and in vitro experiments. In neither instance is there inhibition of either AChE or BuChE.

No determination of peripheral levels of the esterases was made because there already exist data which suggest no inhibition of AChE by botulinum toxin at the motor end plate. Both

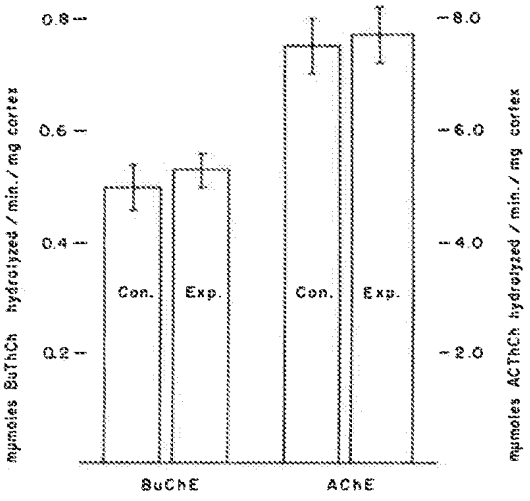

FIG. 2. *Activity of acetylcholinesterase and butyrylcholinesterase in cortices of intoxicated rats. Rats were given lethal doses of botulinum toxin and were decapitated and decorticated. Subsequent assays showed no difference between control and intoxicated rat cortices in their levels of cholinolytic enzyme. The legend on the left is to be used for reading values of butyrylcholinesterase; that on the right, for acetylcholinesterase.*

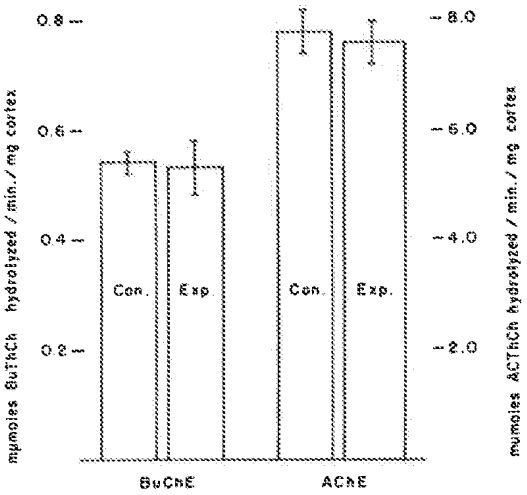

FIG. 3. *Activity of acetylcholinesterase and butyrylcholinesterase from in vitro cortical preparations. Botulinum toxin was added to cortex homogenates of rat brain. The homogenate was assayed for acetylcholinesterase and butyrylcholinesterase, and the values were compared with those from homogenates not incubated with toxin. No difference between control and experimental preparations is evident. The legend on the left is to be used for reading values of butyrylcholinesterase; that on the right, for acetylcholinesterase.*

Brooks (1) and Thesleff (9) have witnessed miniature end plate potentials during paralysis of neuromuscular transmission. Up to the time of transmission failure, there is no increase in amplitude or duration of the miniature end plate potentials, a result that is not compatible with the inhibition of AChE.

With respect to peripheral dysfunction, there is again a mathematical computation of some interest. In the test tube situation, as little as 3.3×10^{10} mg of toxin per 3 ml reportedly caused inhibition. This is equal to nearly 2×10^8 molecules. Zacks et al. (10) have tagged botulinum toxin with ferritin molecules, and, in subsequent electron microscopic studies, localized the toxin-ferritin complex at the neuromuscular junction. In plates 6 and 7 of that publication, it can be seen that there are approximately 10^3 (the approximation means clearly more than 10^2 and clearly less than 10^4) molecules per section. The sections were 0.5-μm thick; therefore, assuming the least stringent conditions, 100 sections would include the entire end plate region. If one assumes that each of the 100 sections contains its nonoverlapping quantum of 10^3 molecules, then the entire end plate region would contain approximately the same number of toxin molecules as the cuvette.

But because of the 3-ml volume of the cuvette and the microscopic volume of the synapse, the toxin is far more concentrated in the synapse. Even if one assumes that the 10^5 molecules in the single section represent all the toxin molecules present at the end plate, the toxin is still considerably more concentrated at the neuromuscular junction than in the cuvette. Under these circumstances, it is curious that no in vivo inhibition of AChE, as evidenced by fasciculation in the poisoned animals, is observed. The curiosity is magnified when it is considered that the cuvette contained orders of magnitude more AChE than is present at the synapse. We thus arrive at the paradox that in the synapse, in which one encounters relatively concentrated toxin and relatively unconcentrated AChE, there is no inhibition; whereas in the cuvette, in which one encounters relatively dilute toxin and relatively concentrated AChE, there is reported inhibition.

From our data and calculations, we draw the following conclusion. There is no in vivo inhibition of AChE by botulinum toxin type A either in the CNS or in the peripheral nervous system. Furthermore, we find the inhibition of AChE by toxin, in a system lacking biological tissue, not to be a universally demonstrable phenomenon. The latter comment is very much in agreement with the recent note of Sumyi and Yocum (8).

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